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Cytokine induction by the immunomodulators imiquimod and S-27609

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Abstract: **Imiquimod (R-837, S-26308) and the analogue S-27609 were evaluated for cytokine induction in human blood cells. Both compounds induced interferon-α (IFN), tumor necrosis factor-α (TNF), interleukin (IL)-1β, and IL-6 with S-27609 being 5 to 10 times more potent. Imiquimod and S-27609 also induced IL-1α, IL-1 receptor antagonist, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), and macrophage inflammatory protein-1α. The profile of cytokines induced by imiquimod and S-27609 was different from those seen with lipopolysaccharide and polyinosinic-polycytidylic acid. Kinetic studies with both imiquimod and S-27609 revealed induction of cytokines as early as 1–4 h after stimulation. Although most of the cytokines produced by S-27609 were secreted, significant concentrations of IL-1α and IL-1β remained intracellular. Monocytes were largely responsible for the cytokines produced. Finally, S-27609-induced mRNA expression for TNF, IFN, and IL-8, and this induction did not require protein synthesis. Taken together, these studies extend previous findings by showing induction of additional cytokines and providing insight into the mechanism of cytokine induction by these molecules.** *J. Leukoc. Biol.* **58: 365–372; 1995.**

Key Words: interferon · interleukin · tumor necrosis factor · lymphokines · lipopolysaccharide · polynucleotides

INTRODUCTION

A new class of immunomodulating agents, represented by the molecule imiquimod (R-837, S-26308), has been found to be an effective antiviral and antitumor agent in animal models. Imiquimod protected guinea pigs from infection by herpes simplex virus (HSV) when given intravaginally, intramuscularly, intraperitoneally, intradermally, subcutaneously (SC), and by mouth (PO) [1, 2]. Imiquimod reduced recurrences induced in guinea pigs by latent HSV infection and, in combination with acyclovir, significantly reduced acute HSV in guinea pigs even after lesions had developed [3–5]. Further studies indicated that imiquimod was active both prophylactically and therapeutically against cytomegalovirus infection in guinea pigs [6] and arbovirus infection in mice [7].

In addition to its antiviral properties, imiquimod inhibited growth of a number of murine tumors including MC-26 colon carcinoma, RIF-1 sarcoma, Lewis lung carcinoma, and a tumor induced by the chemical carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide [8, 9]. Imiquimod not only inhibited growth of the FCB bladder tumor in mice but actually resulted in elimination of tumors from these animals [10].

Imiquimod has no inherent antiviral or antitumor activity in vitro but does stimulate secretion of interferon-α (IFN), which is partially responsible for the in vivo antiviral and antitumor activities. Imiquimod was first found to induce serum IFN in guinea pigs after topical, PO or SC administration [11]. Further studies indicate that imiquimod induces IFN in other animal species including mice, rats, and monkeys [8, 12, 13]. Phase I studies in healthy adult males indicated that imiquimod given PO was capable of inducing detectable concentrations of serum IFN in these volunteers [14–16]. Using antibody neutralization, the type of IFN induced by imiquimod was identified as greater than 97% IFN-α [14]. Indeed, much of the antiviral activity and antitumor activity ascribed to imiquimod was due to the induction of IFN, but other factors seem to contribute as well [2, 4, 8]. More recently, it was demonstrated that imiquimod, its hydroxylated metabolite R-842, and S-27609 stimulated production of a number of other cytokines including tumor necrosis factor-α (TNF), interleukin (IL)-1α, IL-1β, IL-6, and IL-8 in cultures of human peripheral blood mononuclear cells (PBMCs) [17, 18]. Furthermore, TNF and IL-6 along with IFN were detected in the serum of mice following oral administration of imiquimod [19]. The same cytokines were produced in cultures of mouse spleen and bone marrow cells stimulated with imiquimod. In the HSV-infected guinea pig, imiquimod enhanced HSV-specific IL-1 and IL-2 production and increased HSV-specific cell-mediated immune responses [2]. Finally, Kono et al. [20] have demonstrated induction of IL-6 and IL-8 mRNA expression in normal and transformed human keratinocytes. Thus, imiquimod induced a number of different cytokines and other immunomodulating effects, all of which may contribute to its overall biological activity.

The cell responsible for cytokine induction in imidazoquinoline-stimulated human PBMCs has been under investigation. Megyeri et al. [21] have shown that both monocytes and B lymphocytes are capable of secreting

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Abbreviations: FCS, fetal calf serum; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBSS, Hanks' balanced salt solution; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; IL-1 RA, IL-1 receptor antagonist; LPS, lipopolysaccharide; MIP-1α, macrophage inflammatory protein-1α; PBMC, peripheral blood mononuclear cell; poly I:C, polyinosinic-polycytidylic acid; RT-PCR, reverse-transcriptase polymerase chain reaction; RT, room temperature; TNF, tumor necrosis factor-α.

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IFN in response to imiquimod and have further shown induction of mRNA f r IFN, TNF, IL-6, and IL-8 in PBMC cultures. Studies in our laboratory using various separation techniques indicated that monocytes were the cells largely responsible for the cytokines induced by imiquimod and S-27609 [18].

This study was undertaken to further characterize cytokine induction by imiquimod and S-27609 in cultures f human PBMCs. The cyt kine profiles induced by these agents were then compared with those induced by two oth r known immunomodulators, bacterial lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C). We report that imiquimod and S-27609 stimulated production of the cytokines TNF, IFN, IL-1β, and IL-6 in cultures of human PBMCs, with S-27609 being 5- to 10-fold more potent than imiquimod. In addition, S-27609 stimulated IL-1α, IL-1RA, IL-8, IL-10, the colony-stimulating factors (CSF)s granulocyte-macrophage (GM)-CSF and granulocyte (G)-CSF, and macrophage inflammatory protein-1α (MIP-1α). Induction of these cytokines occurs within 1–4 h after treatment with imiquimod and S-27609. Once made, most of the cytokines are secreted rapidly; however, significant concentrations of IL-1α and IL-1β remain inside the cell. Finally, IFN, TNF, and IL-8 mRNA induction by S-27609 was seen in the presence and absence of cycloheximide.

MATERIALS AND METHODS

Isolation of human PBMCs

Whole blood from human volunteers was obtained using EDTA as an anticoagulant after obtaining informed consent. PBMCs were isolated by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO) according to the manufacturer's instructions. Briefly, blood was diluted 1:1 with Hanks' balanced salt solution (HBSS; Sigma) before layering over Histopaque and centrifuging at room temperature (RT) for 30 min at 600*g*. After centrifugation, the mononuclear cells in the plasma-Histopaque interface were transferred to a sterile 50-ml centrifuge tube and PBMCs were washed twice with HBSS by centrifuging for 10 min at 200*g*. Cells were resuspended at a final concentration of 2×10^6 cells/ml in RPMI medium supplemented with 10% fetal calf serum (FCS; Sigma), 2 mM L-glutamine, and penicillin-streptomycin (RPMI complete).

Reagents

The structures of imiquimod (R-837), 1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine; R-842, 4-amino-α,α,2-dimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol; and S-27609, 4-amino-α,α2-trimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol are shown in Figure 1. These compounds are proprietary molecules of 3M Pharmaceuticals and are used as the HCl salt for human PBMC studies. The molecules are dissolved in pyrogen-free water and stored as stock solutions at 4°C for up to 2 months. Bacterial LPS from *E. coli* 055:B5 (Sigma) was dissolved at 1 mg/ml in pyrogen-free water and stored at 4°C for up to 4 months. Poly I:C (Pharmacia LKB, Milwaukee, WI) was prepared by dissolving the molecule in pyrogen-free water at 56°C for 30 min. The solution was then allowed to cool to RT until use. Concentrations of LPS and poly I:C that induced the highest concentrations of cytokines were determined in preliminary studies and these were used throughout this study. The RNA synthesis inhibitor actinomycin D (Calbiochem, La Jolla, CA) was dissolved in water, the protein synthesis inhibitor cycloheximide (Sigma) was dissolved in dimethyl sulfoxide, and both were stored as stock solutions at -20°C.

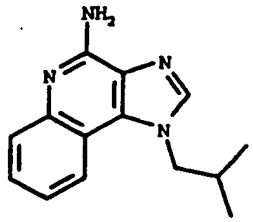
Production of intracellular and extracellular cytokines in human PBMCs

PBMCs (2×10^6 cells/ml) were incubated in six-well tissue culture plates (Corning, Corning, NY) for specified times in a humidified environment at 37°C and 5% CO_2. After incubation, supernatants were collected, filter sterilized, and stored at -20°C for up to 4 weeks until they were assayed for cytokine concentrations. Intracellular fractions were obtained by pelleting the cells at 200*g* for 10 min, removing the supernatant, and resuspending the cells in the original volume of fresh RPMI complete medium. The cells in medium were placed in cryovials, frozen at -20°C, and then subjected to two cycles of freeze-thawing to lyse the cells. The freeze-thawing procedure involved removing the samples from the freezer and allowing them to reach RT. After reaching RT the freeze-thaw cycle was repeated once. The lysates were then sonicated for 5 min and passed through a 22-μm filter to remove debris.

Isolation of monocytes and lymphocytes using countercurrent elutriation

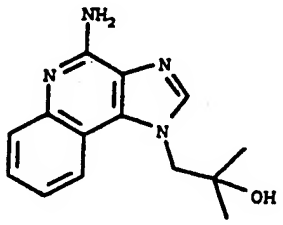
Lymphocyte and monocyte populations were isolated from unseparated PBMCs by countercurrent centrifigual elutriation (CCE) using a Beckman J-6MI centrifuge (Beckman Instruments, Schaumberg, IL) as described by Gerrard et al. [22]. Briefly, tubing and the elutriation chamber were sterilized using 500 ml of 6% H_2O_2 followed by 1000 ml of sterile water and 1000 ml of phosphate-buffered saline (pH 7.4) containing 1 mM EDTA. Flow rate was maintained using a Cole Parmer lab pump (Cole Parmer, Niles, IL). Cells were loaded into the Sanderson chamber at a flow rate of 4 ml/min at a speed of 2400 rpm. The flow

R-837



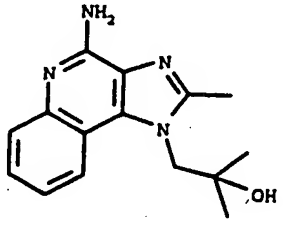
1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine

R-842



4-amino-α,α-dimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethan l

S-27609



4-amino-α,α,2-trimethyl-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol

Fig. 1. Structures of the imidazoquinolinamines: imiquimod, R-842, and S-27609.

rate was increased to 10 ml/min and lymphocytes were collected. This cell population contained less than 1% monocytes as measured by CD14 expression using a FACScan flow cytometer (Becton Dickenson, San Jose, CA). The flow rate was gradually increased 0.5 ml/min until 50% of the cells being eluted were monocytes as measured by forward and side scatter using the FACSCAN. Generally, monocytes were collected at a flow rate of 19 ml/min. The purity of the monocyte population as measured by CD14 expression was greater than 80%. Cell populations were washed once with HBSS (200*g* for 10 min) and then diluted to 2 × 10^6 cells/ml for culture.

Cytokine measurements in human PBMC culture supernatants

Levels of TNF, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, GM-CSF, G-CSF, and MIP-1α were assessed in duplicate for all enzyme-linked immunosorbent assays (ELISAs). TNF and IL-6 concentrations were assessed using ELISA kits purchased from Biosource, Camarillo, CA; IL-1α, IL-1β, IL-1RA, IL-8, GM-CSF, G-CSF, and MIP-1α concentrations were determined using ELISAs purchased from R&D Systems, Minneapolis, MN. Results are presented in pg/ml with coefficients of variation being less than 10% for each sample. Human IFN was measured using a modification of the antiviral assay described by Brennan and Kronenberg [23]. Data are expressed in IFN U/ml using a standard reference preparation of human leukocyte IFN obtained from the National Institutes of Health, Bethesda, MD.

RT-PCR for mRNA induction

Human PBMCs (5 ml at 2 × 10^6 cells/ml in RPMI complete medium) were stimulated for 4 h with S-27609 or LPS. Some of the cultures also received cycloheximide (30 μg/ml). Cells were then washed twice with PBS and mRNA was extracted using an Invitrogen Fast Track mRNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's specifications. Polyadenylated mRNA (0.1 μg) was used as a template for first-strand complementary DNA synthesis using random hexanucleotides (Boehringer Mannheim, Indianapolis, IN) and superscript reverse transcriptase (RT; Gibco-BRL, Gaithersburg, MD). Reactions were performed in a 20-μl volume containing 1 × RT buffer (50 mM KCl, 10 mM Tris-HCl), 5 mM $MgCl_2$, 0.2 mM dNTP, 20 U RNase inhibitor (Gibco BRL), and 50 U of reverse transcriptase. RT-PCR was performed using one-tenth of cDNA for each reaction. Primer sets for glyceraldehyde 3-phosphodehydrogenase, IFN, TNF, and IL-8 as well as cytokine positive controls were purchased from Clontech, Palo Alto, CA. The cDNA was amplified using 0.2 μM 3′ and 5′ primers in 50 μl of PCR mixture containing 1× buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 U AmpliTaq DNA polymerase. Amplification was carried out in a Perkin Elmer/Cetus thermal cycler 9600 (Perkin Elmer Corp., Norwalk, CT). The PCR mixture was heated to 94°C for 2 min followed by 30 cycles with each cycle consisting of 15 s at 94°C, 15 s at 55°C, and 1 min at 72°C. After amplification, the products were separated on 1.8% TAE agarose gels and stained with ethidium bromide (1 μg/ml, Sigma). *Hae*III-digested Phi-χ174 DNA (Gibco BRL) was used for molecular weight markers.

Statistical analysis

Data were analyzed using a paired Student's *t*-test. Results were considered significant only if $P < .05$.

RESULTS

Initial studies compared imiquimod and S-27609 for induction of TNF, IFN, IL-1β, and IL-6 in cultures of human PBMCs. Results in Table 1 demonstrate that both imiquimod and S-27609 induced all of the above cytokines; however, S-27609 is approximately 5–10-fold more potent than imiquimod at stimulating secretion of these cytokines. Production of TNF, IL-1β, and IL-6 by both agents was dose dependent, whereas IFN production stimulat d by imiquimod and S-27609 did not follow a true dose response. Using anti-human neutralizing antibodies to the various types of IFN (α, β, and γ), the type of IFN induced by both imiquimod and S-27609 was found to be greater than 98% IFN-α (data not shown). The concentrations of imiquimod or S-27609 required to produce detectable concentrations of IFN were lower than those required to produce the other three cytokines. TNF, IL-1β, and IL-6 were all produced over the same dose range for imiquimod (1.2–5.0 μg/ml) and S-27609 (0.3–3.0 μg/ml).

The patterns of cytokines released by S-27609 (3 μg/ml) and imiquimod (5 μg/ml) were then compared with the patterns induced by LPS and poly I:C (Table 2). Results showed that S-27609 and imiquimod induced a different pattern of cytokines when compared with LPS or poly I:C. Poly I:C induced the highest concentrations of IFN, with S-27609 and imiquimod inducing only about

TABLE 1. Cytokine Induction by Imiquimod and S-27609 in Cultures of Human PBMCs[a]

	Cytokine concentration (pg/ml) or IFN (U/ml)[b]			
Treatment (μg/ml)	IFN	TNF	IL-1β	IL-6
Medium	0	0	0	0
IMIQ				
(0.3)	0	0	0	0
(0.6)	38	0	0	19
(1.2)	38	30	6	106
(2.5)	38	255	95	457
(5.0)	38	452	365	1,400
609				
(0.03)	0	0	0	0
(0.1)	47	0	0	0
(0.3)	157	276	45	1,430
(1.0)	175	3,514	1,080	4,463
(3.0)	175	8,700	2,870	11,920

[a]Human PBMCs (2 × 10^6 cells/ml) were incubated for 24 h at 37°C with various concentrations of imiquimod (IMIQ) or S-27609 (609). Supernatants were collected, filter sterilized, and stored at -20°C until they were assayed for cytokines.

[b]IFN concentrations were assayed by bioassay and results are presented in U of IFN/ml and are taken from a single experiment. TNF, IL-1β, and IL-6 concentrations were determined by ELISA and are presented in pg/ml.

TABLE 2. Cytokine Induction by S-27609, Imiquimod, LPS, and Poly I:C in Human PBMCs[a]

Treatment	Cytokine concentration (pg/ml) or IFN concentration (U/ml)[b]									
	IFN	TNF	IL-1α	IL-1β	IL-1RA	IL-6	IL-8	IL-10	G-CSF	GM-CSF	MIP-1α
Medium[c]	0	9 ± 5	0	5 ± 2	751 ± 251	45 ± 27	2,605 ± 1,007	7 ± 3	2 ± 1	0.4 ± 0.3	65 ± 34
Imiquimod (5)	347 ± 98*	75 ± 19*	5 ± 2	171 ± 57*	8,174 ± 1,740*	853 ± 227*	16,741 ± 7,165	70 ± 19*	14 ± 6	10 ± 2*	2,783 ± 2,008*
S-27609 (3)	415 ± 117*	825 ± 150*	141 ± 25*	2,111 ± 414*	15,956 ± 2,535*	4,871 ± 910*	166,825 ± 9,382*	519 ± 101*	224 ± 67*	40 ± 11*	15,615 ± 2,937*
LPS (1)	64 ± 19*	762 ± 108*	681 ± 130*	2,877 ± 611*	9,782 ± 1,390*	9,748 ± 1,630*	446,750 ± 55,469*	1,998 ± 222*	6,454 ± 1,298*	998 ± 310*	27,164 ± 7,013*
Poly I:C (50)	2,079 ± 223*	48 ± 12	18 ± 7	317 ± 164	6,839 ± 1,207*	294 ± 113*	7,700 ± 5,416	60 ± 1	2 ± 2	5 ± 1	198 ± 63

[a]Human PBMCs were stimulated for 24 h with imiquimod (5 μg/ml), S-27609 (3 μg/ml), LPS (1 μg/ml) or poly I:C (50 μg/ml) after which the supernatants were collected, filter sterilized, and then analyzed for various cytokine levels. Data are presented as the mean ± SEM of four to six experiments. *$P < .05$ compared with the medium control.
[b]IFN levels were detected by bioassay and are presented in U/ml. All other cytokine levels were determined using ELISA and are presented in pg/ml.
[c]Control cultures contained cells incubated in RPMI complete without any stimulus.

20% as much as poly I:C. LPS was the least effective inducer of IFN; however, it was the most potent inducer of IL-1α, IL-6, IL-8, IL-10, G-CSF, GM-CSF, and MIP-1α. S-27609 produced 2–4 times less IL-1α, IL-6, IL-8, IL-10, and MIP-1α and 30–50 times less CSFs than LPS. S-27609 and LPS induced similar concentrations of TNF and IL-1β and higher concentrati ns of IL-1RA compared with LPS. Imiquimod and poly I:C were less effective at inducing the ILs and CSFs wh n compared with either LPS or S-27069; h wever, concentrations of IL-1 RA induced by imiquimod and poly I:C were similar to those induced by S-27609 and LPS. Imiquimod stimulated higher concentrations of IL-6, IL-8, and MIP-1α than poly I:C but did not significantly induce IL-1α. Poly I:C did not induce significant production of GM-CSF, G-CSF, or MIP-1α. These studies clearly show differences in cytokine profiles induced by the imidazoquinolines and the other cytokin inducers LPS and poly I:C.

The kinetics of cytokine induction in human PBMCs by S-27609 (3 μg/ml) were evaluated (Table 3). TNF induction by S-27609 was observed as early as 1 h after incubation with amounts peaking at 4–6 h. Concentrations remained elevated over the next 18 h of culture. Significant IFN was observed 2 h after S-27609 stimulation with concentrations peaking at 6 h and remaining constant for the next 18 h. Significant elevations of IL-1β and IL-6 concentrations induced by S-27609 were not seen until 4 h after stimulation. Unlike IFN and TNF, IL-1β and IL-6 concentrations continued to rise throughout the entire culture period, with the highest concentrations at 24 h. Significant IL-8 induction by S-27609 was observed 4 h after stimulation with concentrations rising rapidly until 6 h after stimulation. During the next 16 h, IL-8 concentrations increased fourfold. IL-8 concentrations were detected in untreated cultures with the kinetics being quite similar to those seen in S-27609–stimulated cultures. Kinetics of IFN, TNF, IL-1β, IL-6, and IL-8 induction by imiquimod were also evaluated in human PBMCs (Fig. 2). Results were similar to those seen with S-27609.

The next set of studies evaluated intracellular and extracellular concentrations of cytokines induced by S-27609 after 24 h of stimulation of human PBMCs (Tabl 4). Concentrations of IFN, TNF, IL-6, and IL-8 found inside the cell were less than 10% of the total cytokine that was secreted in response to S-27609 or LPS. In contrast, significant concentrations of IL-1α and IL-1β remained inside the cell when they were stimulated with S-27609 (45%) or LPS (48%). Approximately 15% and 26% of the IL-1β remained intracellular upon stimulation with S-27609 and LPS, respectively.

Studies implicating both monocytes and B cells as the cells producing IFN in response to imiquimod have been reported [18, 21]. We evaluated monocytes and lymphocytes isolated by CCE for their ability to produce IFN, TNF, and IL-6 in response to S-27609, imiquimod, LPS, and poly I:C (Table 5). Imiquimod, S-27609, and LPS produced similar concentrations of IFN in PBMCs and monocyte-enriched cultures. Untreated lymphocytes had elevated IFN activity; however, IFN concentrations induced by imiquimod, S-27609, and LPS were significantly lower than those seen in unseparated and monocyte-enriched subpopulations. Poly I:C induced high concentrations of IFN in all three cell populations.

Imiquimod, S-27609, and LPS induced significant c ncentrations of TNF in monocyte-enriched but not lymphocyte-enriched cultures. Concentrations of TNF

TABLE 3. Kinetics of Cytokine Induction in Human PBMCs by S-27609[a]

	Cytokine concentration (pg/ml) or IFN concentration (U/ml)[b]									
	IFN (U/ml)		TNF (pg/ml)		IL-1β (pg/ml)		IL-6 (pg/ml)		IL-8 (pg/ml)	
Time (h)	Medium[c]	S-27609	Medium	S-27609	Medium	S-27609	Medium	S-27609	Medium	S-27609
1	0	1 ± 0.7	2 ± 1	64 ± 42	0.7 ± 0.7	0.7 ± 0.7	1.4 ± 1.3	6.3 ± 4.6	17 ± 14	187 ± 134
2	0	36 ± 15	2 ± 2	416 ± 110*	0.7 ± 0.7	7.2 ± 7.2	4.9 ± 3.1	15.6 ± 9.1	138 ± 56	1,478 ± 603
4	0	153 ± 45*	6. ± 4	1,556 ± 190*	0.7 ± 0.7	515 ± 276*	2.4 ± 1.7	1,775 ± 566*	1,151 ± 830	11,850 ± 1,074*
6	0	260 ± 94*	7. ± 5	3,400 ± 1,755*	0.8 ± 0.2	929 ± 559*	18 ± 18	4,734 ± 577*	2,962 ± 1,310	40,695 ± 4,655*
8	0	245 ± 72*	17 ± 13	2,577 ± 704*	0.7 ± 0.6	1,803 ± 595*	6.8 ± 6.8	7,137 ± 1,191*	974 ± 1,949	85,180 ± 8,672*
24	0	303 ± 96*	10 ± 8	2,067 ± 407*	0.5 ± 0.3	2,365 ± 941*	4.8 ± 4.4	14,760 ± 2,626*	7,025 ± 2,495	181,400 ± 26,588*

[a]Human PBMCs were stimulated for various times with S-27609 (3 μg/ml), after which the supernatants were collected, filter sterilized, and then analyzed for various cytokine levels. Data are presented as the mean ± SEM of six experiments. *$P < .05$ compared with the appropriate medium control.

[b]IFN levels were detected by bioassay and are presented in U/ml. All other cytokine levels were determined using ELISA and are presented in pg/ml.

[c]Control cultures were cells incubated in RPMI complete without any stimulus.

stimulated by S-27609 and LPS were slightly higher in monocyte cultures than in unseparated PBMC cultures. Poly I:C was ineffective at inducing significant TNF in all three populations. All four stimuli induced significant concentrations of IL-6 in unseparated PBMCs and monocyte-enriched cultures but not in lymphocyte-enriched cultures. These data further support the important role of monocyte/macrophages as the major cell responsible for cytokine induction by the imidazoquinolines.

To determine whether both RNA synthesis and protein synthesis were required for cytokine induction by S-27609, cells were stimulated in the presence or absence of the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D (Fig. 3). Results are presented as the mean of three experiments and demonstrate that cycloheximide and actinomycin D significantly inhibit S-27609–induced TNF, IL-1β, IL-6, and IL-8. Similar observations were made using LPS and imiquimod as the stimuli (data not shown).

Although these data suggest that both protein synthesis and RNA synthesis are required for imidazoquinoline-induced cytokine production, they do not demonstrate that de novo synthesis is required. The final set of studies evaluated cytokine mRNA induction by S-27609. Human PBMCs were stimulated with S-27609 or LPS in the presence or absence of cycloheximide for 4 h, after which mRNA was isolated and analyzed by RT-PCR for induction of IFN-α, TNF, and IL-8 mRNA expression (Fig. 4). S-27609 induced increases in IFN-α, TNF, and IL-8 mRNA expression when compared with untreated cells. Induction of these mRNAs by S-27609 was also potentiated by cycloheximide, indicating that protein synthesis was not required for mRNA induction. LPS induced increases in TNF and IL-8 but not IFN-α mRNA expression when compared with untreated cells. Cycloheximide again augmented the expression of TNF and IL-8 mRNA in response to LPS. Interestingly, cycloheximide induc d increases in TNF and IL-8 mRNA expression.

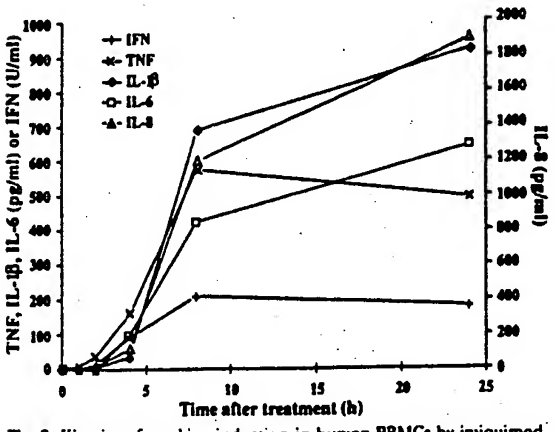


Fig. 2. Kinetics of cytokine induction in human PBMCs by imiquimod. Human PBMCs (2×10^6 cells/ml) in RPMI complete were incubated for 1, 2, 4, 8, and 24 h, after which culture supernatants were collected, filter sterilized, and assayed for IFN, TNF, IL-1β, IL-6, and IL-8. Results are presented in U/ml for IFN-α; all other cytokine concentrations are presented in pg/ml. Results are presented from a representative experiment that was repeated two separate times.

DISCUSSION

Imiquimod and the analogue S-27609 are low-molecular-weight imidazoquinolines that exhibit potent antiviral and antitumor activities in a number of animal models when given either prophylactically or therapeutically [1–10]. Their ability to induce IFN in a number of species including humans is well documented and is considered to be a major mediator of this class's biological activities [11–16]. We have demonstrated that imiquimod also induces other cytokines in mice [19] and human PBMC cultures [17, 18].

Imiquimod and S-27609 induce the same cytokine profile in human PBMCs; however, S-27609 is 5–10 times more potent. Weeks and Gibson [17] have shown that imiquimod and the imiquimod metabolite R-842 had similar potency. Taken together, these results indicate that addition of a methyl group at the 2 position increases the potency of these molecules. A more complete structure-activity relationship of this class of compounds will be reported (J.F. Gerster, manuscript in preparation).

It appears that concentrations of both imiquimod and S-27609 required to induce IFN in human PBMCs are lower than those required to induce the other cytokines. This could be due to preferential induction of IFN at

TABLE 4. Intracellular and Extracellular Production of Cytokines by S-27609 and LPS[a]

	Stimulant			
	S-27609 (3 μg/ml)		LPS (1 μg/ml)	
	Intracellular[b]	Extracellular	Intracellular[c]	Extracellular
[illegible] (U/ml)	3 ± 0.5	223 ± 111*	8.5 ± 5	46 ± 32
[illegible] (pg/ml)	89 ± 18	1,832 ± 178*	46 ± 24	1,247 ± 330*
[illegible] (pg/ml)	252 ± 39	509 ± 82*	470 ± 45*	512 ± 82*
[illegible] (pg/ml)	424 ± 135*	2,477 ± 23*	1,222 ± 386*	3,375 ± 875*
[illegible] (pg/ml)	9 ± 9	8,201 ± 1,050*	24 ± 24	7,675 ± 1,406*
[illegible] (pg/ml)	7,725 ± 3,650	132,515 ± 31,376*	11,428 ± 4,492	188,275 ± 28,978*

[a] Human PBMCs were stimulated for 24 h with S-27609 (3 μg/ml) or LPS (1 μg/ml), after which the supernatants were collected, filter sterilized, [illegible] analyzed for various cytokine levels. Data are presented as the mean ± SEM of four experiments. *$P < .05$ compared with the medium [illegible]

[b] IL-1 levels were detected by bioassay and are presented in U/ml. All other cytokine levels were determined using ELISA and are presented [illegible]

[c] Intracellular cytokine concentrations were determined after cells underwent two cycles of freeze-thawing.

[illegible] concentrations; alternatively, it may be that the bioassay is more sensitive at detecting IFN than the ELISA [illegible] for the other cytokines. The induction of IFN by imiquimod and S-27609 does not follow a true dose-response relationship, whereas that of TNF, IL-1β, and IL-6 [illegible]

Studies comparing cytokine profiles for the different immunomodulators demonstrate that imiquimod and S-27609 have different patterns. Imiquimod and S-27609 are more effective than LPS but less effective than poly I:C at inducing IFN. Weeks and Gibson [17] reported that poly I:C was unable to induce IFN in human PBMCs. The difference in results can be attributed to the serum used in culturing the cells (FCS versus autologous serum). Human serum is known to contain nucleases that degrade the poly I:C [24]. Others have also shown little or no induction of IFN by LPS unless monocytes are first primed with IFN-γ or GM-CSF [25].

All four immunomodulators were potent inducers of IL-1 RA, with S-27609 inducing the highest concentrations. Concentrations of S-27609 that induce IL-1 RA and IL-1 (>0.1 μg/ml for S-27609 and >0.6 μg/ml for imiquimod) are ineffective at inducing significant concentrations of IL-1. The induction of IL-1 RA is probably due to both direct induction and indirect induction by IL-1 or IL-6 [26–29]. Studies designed to address this hypothesis are in progress.

S-27609 and LPS induced similar concentrations of TNF, IL-1, IL-6, IL-8, IL-10, and MIP-1α. Both imiquimod and poly I:C induced only low concentrations of IL-1, TNF, IL-8, IL-10, and MIP-1α when compared with either S-27609 or LPS. Imiquimod induced slightly higher concentrations of IL-6, IL-8, and MIP-1α than poly I:C; however, both were marginally effective at inducing G-CSF and GM-CSF. Weeks and Gibson [17] reported that imiquimod did not induce GM-CSF; however, we have detected low but significant concentrations of this cytokine in our culture systems. The differences seen may be due to slight variations in culture conditions as well as the donor cells used for induction. In comparison to imiquimod, S-27609 induced higher concentrations of both GM-CSF and G-CSF; however, concentrations were still 30–50 times less than those produced in response to LPS.

Kinetics of cytokine induction by S-27609 and imiquimod are similar. TNF is first detected as early as 1 h after treatment (this was not significant). IFN, IL-1, IL-6, and IL-8 are detected as early as 2 h after stimulation with either S-27609 or imiquimod. The CSFs are first seen 4 h after treatment with S-27609 (data not shown). Others have shown similar kinetics for TNF, IL-6, and IL-8 production utilizing LPS and human whole blood [30–32]. Whereas TNF and IFN concentrations plateau 8 h after stimulation, concentrations of IL-1β, IL-6, and IL-8 con-

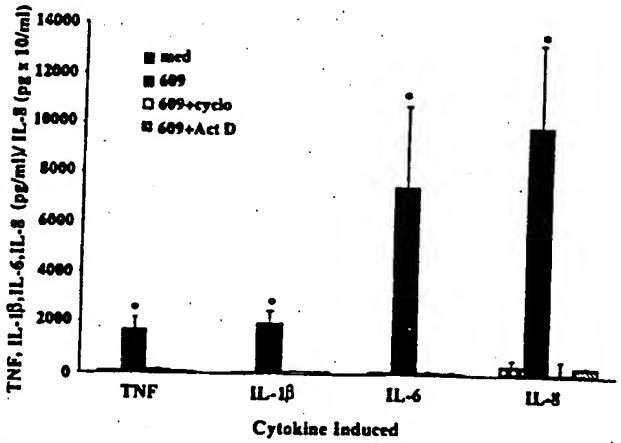


Fig. 3. Effects of cycloheximide and actinomycin D on cytokine induction by S-27609 and LPS. Human PBMCs (2×10^6 cells/ml) in RPMI complete were incubated for 24 h with 3 μg/ml S-27609 in the presence of various concentrations of either cycloheximide or actinomycin D. Supernatants were then collected, filter sterilized, and assayed for TNF-α, IL-1β, IL-6, and IL-8. Results are presented in pg/ml and data are presented as the mean of three individual experiments ± SEM. IL-8 concentrations are presented in pg × 10/ml. *$P < .05$.

TABLE 5. Monocytes but Not Lymphocytes Produce Cytokines in Response to S-27609 or Imiquimod[a]

	Cytokine concentration (pg/ml) or IFN concentration (U/ml)[b]								
	IFN			TNF			IL-6		
Treatment	PBMC	Lymph	Mono	PBMC	Lymph	Mono	PBMC	Lymph	Mono
Medium	0	67 ± 29	18 ± 7	10 ± 10	6 ± 6	19 ± 18	70 ± 61	41 ± 9	22 ± 11
Imiquimod (5)	150 ± 95	1 ± 1*	67 ± 40	44 ± 13	9 ± 9*	42 ± 24	1,093 ± 432	13 ± 4*	1,267 ± 428
S-27609 (3)	164 ± 89	9 ± 4*	197 ± 121	356 ± 128	0*	954 ± 472	3,854 ± 1,056	36 ± 6*	5,984 ± 2,024
LPS (1)	48 ± 32	1 ± 1*	51 ± 30	832 ± 204	0*	1,341 ± 589	10,482 ± 1,044	21 ± 6*	13,000 ± 2,000
Poly I:C (50)	3,395 ± 1,122	1,386 ± 563	2,474 ± 404	12 ± 12	15 ± 15	32 ± 26	326 ± 206	10 ± 4*	308 ± 123

[a]Human PBMCs were stimulated for 24 h with S-27609 (3 μg/ml) or LPS (1 μg/ml), after which the supernatants were collected, filter sterilized, and then analyzed for various cytokine concentrations. Data are presented as the mean ± SEM of four experiments. *$P < .05$ compared with similarly treated unseparated PBMC.

[b]IFN was detected by bioassay and are presented in U/ml. Other cytokines were determined using ELISA and are presented in pg/ml. Lymph indicates the lymphocyte-enriched population and Mono indicates the monocyte-enriched population.

tinue to rise during the last 16 h. As TNF and IL-1 have been shown to induce IL-1, IL-6, and IL-8 [31-34], the later elevations may be due to indirect induction by TNF or IL-1.

The results evaluating intracellular and secreted cytokine concentrations were not surprising. More than 90% of all of the cytokines, except for IL-1α and IL-1β, were secreted. On the other hand, approximately 50% of the IL-1α and 15-25% of the IL-1β remained inside the cell upon stimulation with S-27609 or LPS. Previous studies by others using LPS show that IL-1α remains largely intracellular [35, 36].

Studies by Megyeri et al. [21] have implicated both B cells and monocytes as the cells responsible for IFN release by imiquimod. Studies in our laboratory using imiquimod and S-27609 point to a strong role of monocytes in the release of IFN and other cytokines [18]. These studies were based highly on elimination of populations responsible for cytokine induction rather than on positive selection. In this study we used positive selection of both lymphocytes and monocytes by CCE to evaluate cytokine induction by imiquimod and S-27609. Results showed induction of all cytokines evaluated to be in the monocyte-enriched fraction. On average, this population contained greater than 85% monocytes as measured by CD14 expression and less than 5% lymphocytes. Thus, it appears that monocytes are the major cells secreting cytokines in response to imiquimod and S-27609.

Generally, with monocyte secretion of cytokines, de novo synthesis is required rather than release of stored proteins as is observed for TNF secretion by mast cells [37]. The induction of all of the cytokines produced in response to S-27609 and LPS was inhibited by addition of actinomycin D or cycloheximide. IFN induction was not evaluated in the presence of actinomycin D and cycloheximide because these agents would negatively affect the

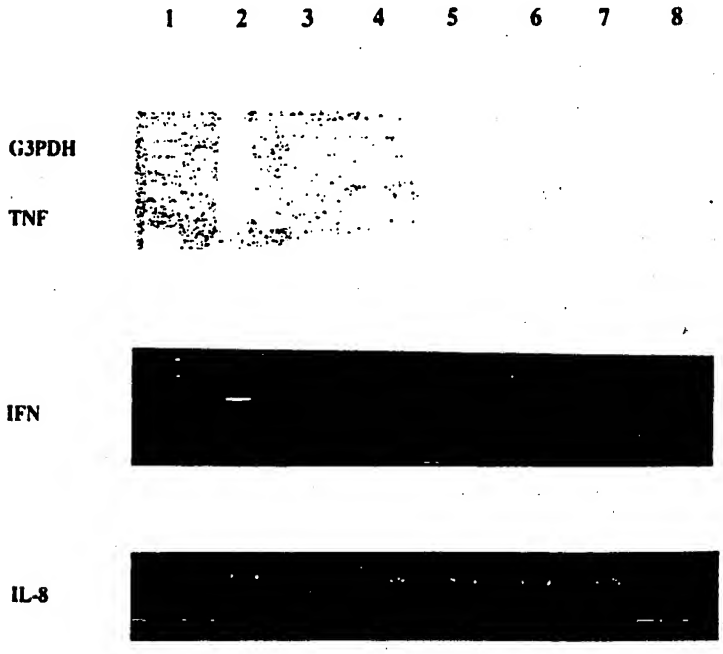


Fig. 4. IFN, TNF, and IL-8 mRNA induction by S-27609. Human PBMCs were stimulated for 4 h, after which RNA was extracted and amplified using RT-PCR. Samples were separated by agarose gel electrophoresis. Lane 1, molecular weight markers; lane 2, positive control cDNA; lane 3, unstimulated PBMCs; lane 4, S-27609 (3 μg/ml)-treated PBMCs; lane 5, S-27609 (3 μg/ml) + cycloheximide (30 μg/ml)-treated PBMCs; lane 6, LPS (1 μg/ml)-treated PBMCs; lane 7, LPS (1 μg/ml) + cycloheximide (30 μg/ml)-treated PBMCs; lane 8, cycloheximide (30 μg/ml)-treated PBMCs.

IFN bioassay. Further studies evaluating mRNA expression clearly demonstrated induction of IFN-α, TNF, and IL-8 mRNA by S-27609. Induction was also seen in the presence of cycloheximide, indicating that protein synthesis was not required for induction and that mRNA induction occurs through activation of already present transcription factors. Similar results were obtained by Megyeri et al. [21] when they evaluated induction of IFN, TNF, IL-6, and IL-8 mRNA by imiquimod. The stimulation of IL-1, IL-6, IL-8, and TNF expression by imiquimod occurs in part through activation of NF-κB [21]. However, IFN-α genes lack an NF-κB binding site, indicating that other transcription factors are needed to activate these genes. Indeed, imiquimod stimulates protein binding to the 4F1 site found in the promoter region of the IFN-α genes [21]. It is postulated that S-27609 will behave in a similar manner to imiquimod.

In conclusion, this study extends previous findings demonstrating that imiquimod and S-27609 induce a cascade of cytokines including TNF, IFN, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, IL-10, MIP-1α, GM-CSF, and G-CSF. Induction of these cytokines is detected 1–4 h after stimulation and follows a definite pattern of appearance. Induction of cytokines by S-27609 correlates with increases in cytokine mRNA expression. Finally, the imidazoquinolines can no longer be thought of as just IFN inducers. They must also be thought of as immunomodulators with broader effects that result in a number of cytokines whose further immunological effects act in concert to contribute to the overall biological activity of this class of agents.

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